NIGERIAN VETERINARY JOURNAL
ISSN 0331-3026
Nig. Vet. J., July 2024 Vol 45 (2): 26-38.
https://dx.doi.org/10.4314/nvj.v45i2.3 ORIGINAL ARTICLE

Serological and Molecular Detection of Low Pathogenic Avian Influenza Subtype H9N2 in Commercial Poultry Flock in Kaduna and Kano State Nigeria.

Nicodemus Mkpuma[1*], Lukman Muftahu Oyedeji [2], Alexander Oluyinka Akintule [2], Chinonyerem N. Chinyere [1], Adeyinka Olamide Agbato [3], Inuwa Bitrus [1], Shamsuddeen Shehu [4], Kayode A. Olawuyi [1], Donna Bala Tige [2], Dorcas A. Gado [1], Clement A Meseko [1]

[1] Regional Laboratory for Animal Influenza and other Transboundary Animal Diseases, National Veterinary Research Institute, Vom, Plateau state, Nigeria. National. [2]Kaduna Poultry Disease Laboratory (Olam Grains), Kaduna State, Nigeria. [3]Animal Care Services Konsult Technical Laboratory, Abuja, Nigeria. [4]Ambuvet Konsult, Kaduna State, Nigeria. *Corresponding author: Email: nicodemusmkpuma@gmail.com; Tel No: +2348106465204

SUMMARY

The poultry business in Nigeria has been severely affected by highly pathogenic avian influenza (HPAI), particularly the H5 variant. Unlike H5 and H7 subtypes, low pathogenic strains like the H9N2 are not notifiable to the OIE. However, in many instances in the West African sub-region, this strain poses a threat to chicken and turkey farming. In the present study, reports of mortality in commercial layer chicken with clinical indications and lesions of Newcastle disease and *E. coli* septicemia from two farms in Kano and Kaduna states. Ten tracheal, cloacal swabs, and blood samples were collected from moribund and dead birds on each of these farms and sent to the lab for analysis. Using the Enzyme Linked Immunosorbent Assay, antibodies against the H9 subtypes of the AIV were found in 85 percent (17/20) of the sera. Low pathogenic avian influenza subtype H9N2, *Escherichia coli* and *Klebsiella spp* were detected in six samples by RT-qPCR and Microbiology respectively as well as virus isolation in 9-11day old specific antibody negative embryonated chicken egg. Hyperaemia, oedema, necrosis and loss of cilia on the epithelial lining of the trachea with heterophils and lymphocytes infiltration of the lamina propria were observed in all the samples. These findings confirmed the presence and circulation of the low pathogenic subtype H9N2 in commercial layer flock; though the virus is biologically low pathogenic, co-infection with other pathogens could exacerbate infection and cause severe economic losses. We suggest that all subtypes of avian influenza virus be monitored at all times across the country.

Key words: Avian influenza, H9N2 ELISA, Nigeria, RT-qPCR, virus isolation.

INTRODUCTION

The devastating economic losses associated with the outbreak of highly pathogenic avian influenza (HPAI) in poultry is well-known among poultry farmers in Nigeria. The Nigerian poultry sector provide over 25 million jobs (direct and indirect)

to support livelihoods of millions of families in the country (Bello *et al*., 2015). Outbreaks of avian influenza has however, continued to threaten this important sector of the economy.

Avian Influenza is caused by Influenza A virus, an enveloped single stranded RNA viruses of the family Orthomyxoviridae (MacLachlan *et al*., 2017). A classification system was developed for influenza viruses because of the practical need to assess the risk represented by the emergence of new variant viruses, and the need to determine herd or population immunity against previously circulating strains so that vaccine requirements can be assessed. Based on this system, Influenza A viruses are categorized into 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes, although recently described influenza A viruses may increase the number of hemagglutinin subtypes (MacLachlan *et al*., 2017). These viruses are named by the combination of H and N they express and so far, all influenza A subtypes in most possible combinations have been isolated from avian species (Capua and Alexander, 2009). Based on the degree of damage avian influenza viruses cause to the poultry industry, they are categorized into two groups; Low pathogenic (LPAI) strain which typically cause few or no clinical signs in poultry and; highly pathogenic (HPAI) strain, which cause severe clinical signs and potentially high mortality rates among poultry (WHO, 2002). The OIE terrestrial animal health code has adopted 2 criteria for determining the pathogenicity of an avian influenza virus. The first criteria classify any influenza A virus with intravascular pathogenicity index (IVPI) above 1.2 as highly pathogenic avian influenza virus. The second criteria consider all H5 and H7 viruses in chicken with amino acid sequence of the connecting peptide of the haemagglutinin glycoprotein similar to that observed for other HPAI isolates as HPAI viruses (WHO, 2002).

To date, naturally occurring highly pathogenic influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes (WHO, 2002; Swayne and Pantin-Jackwood, 2008; Swayne *et al*., 2020).

Highly pathogenic avian influenza especially the H5 subtype has been the focus in the poultry subsector in Nigeria probably due to the devastating economic impact of repeated outbreaks from these strains particularly the H5N1 and its public health significance. However, H9N2 viruses capable of causing moderate to severe disease in chickens, but still meeting the definition of LPAI virus, has emerged, and spread widely in Asia, the Middle East, North and West Africa (Swayne *et al*., 2020). Despite poor diagnostic capabilities and reporting system in most African countries, H9N2 viruses has been reported in chicken or turkey in countries like Tunisia (Tombari *et al*., 2011), Egypt (Abdel *et al.,* 2016), Morocco (El Houadfi *et al*., 2016), Burkina Faso (Zecchin *et al.,* 2017), Libya (Nagy *et al*., 2017), Algeria (Peacock *et al*., 2019), Ghana (Awuni *et al*., 2019), Kenya (Kariithi *et al*., 2020), Benin, Togo and Uganda (Maxime *et al*., 2021), South Africa in Ostrich (Celia *et al*., 2010) as well as multiple human cases in Senegal (Mamadou *et al*., 2017). This is not without most countries having the potential for endemicity.

Other LPAI such as H5N2 and H7 has been reported in LBM Nigeria through molecular and serological identification respectively (Coker *et al*., 2014; Oluwayelu *et al*., 2017; Chinyere *et al*., 2020; Abiayi *et al*., 2021).

In 2019, Nigeria reported its first case of LPAI H9N2 belonging to the G1 lineage in LBM, which has zoonotic potential and grouped with other LPAI H9N2 discovered in Africa (Suleiman *et al.*, 2021).

Until now, outbreak of avian influenza associated with the H9N2 subtype has not been detected in commercial poultry in Nigeria. Between February 2020 and March 2021, two commercial chicken layer farms with similar clinical manifestation and varied mortality rates were monitored. Avian influenza H9N2 virus and antibodies against the virus were detected in each of the farm during the course of the outbreaks of the disease. This article provides a description of these outbreaks and of viral recovery from the infection.

MATERIALS AND METHODS

Description of Outbreaks

In December 2019, there was a spike in cases of what was clinically described by Veterinarians as Newcastle disease - *E. coli* septicaemia syndrome in laying birds in Nigeria. These symptoms were often observed following administration of Newcastle disease vaccine (Lasota) booster. From January 2020, to April 2021, multiple cases with similar presentations were reported to a private laboratory "Kaduna Poultry Disease Laboratory, Kaduna State, Nigeria". Outbreaks from 2 different multiage chicken layer flocks were described here (Figure 2).

The 2 farms monitored were in Kaduna and Kano States Nigeria, housing from 4 to 6 flocks of 18 to 59 weeks old commercial layers. All flocks are reared in cages and fed commercial layer mash. Lasota vaccine booster is a monthly routine in these farms and was administered 3 to 5 days before the onset of illness. Additional history revealed that birds manifested reduced feed and water intake, mild respiratory distress including rales, sneezing and gasping, and later drop in egg production, increased cracked and soft-shelled eggs and greenish yellow watery faeces. After about 10 days, recovery was observed starting with improvement in appetite and slow increase in egg production. The disease will often appear in one pen and eventually spread to others. Morbidity ranged from 18% and 40% Mortality rate was 0.2 % to 3.5 % depending on severity of secondary bacterial infection (Table I).

More severe consequences were associated with drop in egg production with loss of up to 40% production during the acute stage of the disease. Pattern of drop in egg production as observed in the farms monitored is shown in figure 2.

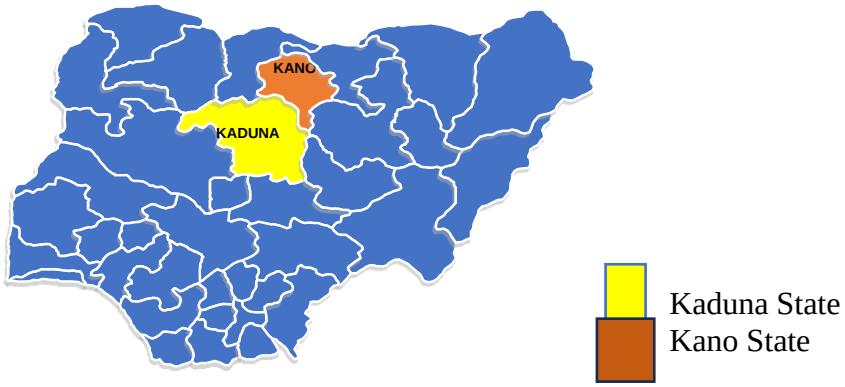


Figure 1: Map of Nigeria showing location of farm monitored

Sera were harvested from blood samples submitted with birds.

Farm location	Age of birds (wks)	Flock size	(%) morbidity	(%) mortality
Kaduna (Farm A)	18-43	15,000	40	3.5
Kano (Farm B)	46-63	40,000	18	0.2

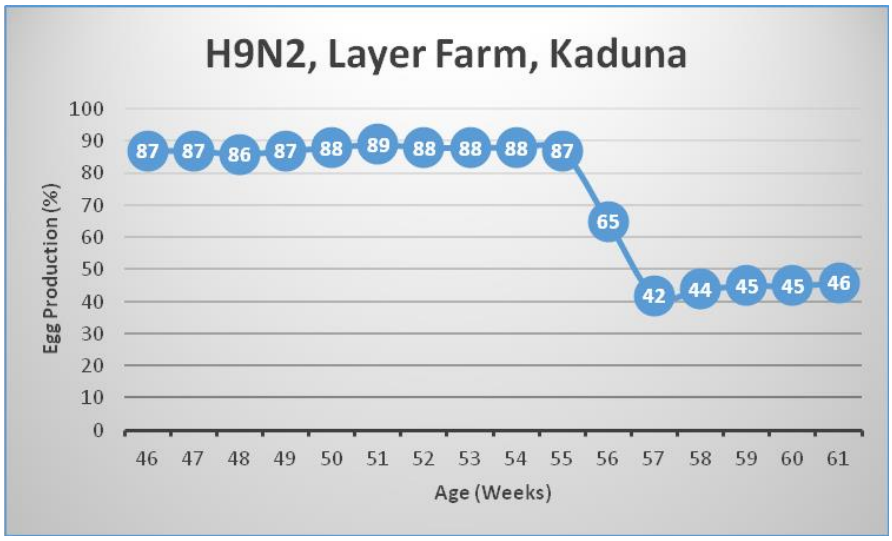


Figure 2: Egg-laying curve in layers from farm A infected with H9N2 Avian Influenza virus.

Necropsy and histopathology

Moribund and dead birds were submitted to the Kaduna Poultry Disease Laboratory, Kaduna State, Nigeria for analysis. Moribund birds were humanely euthanized. Four to seven dead birds were examined. Tracheal samples were collected and fixed in 10 % buffered neutral formalin for 48 hours, dehydrated in graded alcohols, cleared with xylene, and infiltrated and embedded in paraffin wax. Embedded tissues were then sectioned at 5µ and stained with hematoxylin and eosin.

Serology

Twenty (20) sera samples which serves as representative from the two were analysed using IDvet Avian Influenza indirect and competitive antibody capture ELISA for Avian influenza H5, H7 and H9 virus subtypes (IDvet, Grabels, France) according to manufacturers instruction.

Molecular analysis and virus isolation

Sample collection

Sterile plastic-shafted flocked swabs were used to collect tracheal and cloacal swabs (Puritan Medical, Guilford, ME). A total of twenty birds were swabbed from each farms. Each swab was placed in individual 1.8 ml Corning® cryogenic

vials (Corning Inc., Corning, New York, USA) containing 1.5ml of viral transport media supplemented with antibiotics and antifungals (penicilin, streptomycin, gentamycin and amphotericin B (WHO, 2006).
The swabs were kept on ice block and immediately transported to the Regional Laboratory for Avian Influenza and Other Transboundary Animal Diseases, National Veterinary Research Institute, Vom, Nigeria for molecular diagnostics, viral isolation and characterization.

RNA extraction

Nucleic acid was extracted from tracheal and clocal swabs,using Qiagen RNeasy Mini Kit, a high Pure RNA extraction Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. RNA was eluted in a final volume of 50 µl and stored at -80°C.

Quantitative (real time) PCR assay

The RNA were screened for matrix (M) gene using real-time RT-PCR assays on a Rotor G (Qiagen Co, CA) in a 25 µL reaction mixture containing 5 µL of RNA, 1.5 µL of each primer, M+25 forward and M-124 reverse (5 µM), 2.5µL of M+64 FAM probe (1 µM), 12.5 2X RT-PCR master mix, 0.2 µL enzyme mix (Quantitect multiplex), and 1.8 µL of Rnase-free water with the following cycling conditions: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45s and 60°C for 45s (Spackman *et al*., 2002). Those with CT value of 35 and below were further subtyped according to (Isabella *et al*., 2008) in a 25 µL reaction mixture containing 5 µL of RNA, 1.5 µL of each primer, H9 Forward and H9 Reverse (5 µM), 3.75µL of H9 FAM probe (1 µM), 12.5 2X RT-PCR master mix, 0.2 µL enzyme mix (Quantitect multiplex), and 0.55 µL of Rnase-free water with the following cycling conditions: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45s and 54°C for 45s. All the primers and probe were synthesed by a private company "Macrogen the Netherland"

Virus isolation

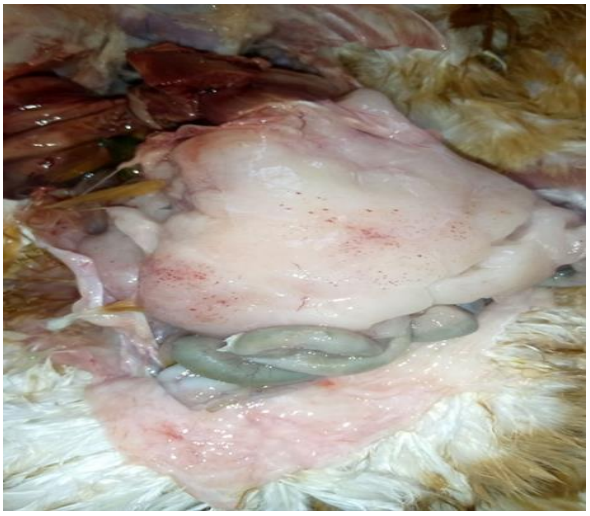
Swabs in 0.2 ml viral transport media (VTM) were inoculated into allantoic fluid of 9- 11-day old specific antibody negative (SAN) embryonated chicken eggs using sterile 20-gauge syringe. Inoculation hole was closed by paraffin. Embryonated eggs were incubated at 38°C, 50 – 60 % relative humidity for 24-72 hours. Inoculated eggs were candled at least once a day to identify eggs with dead embryos. Dead embryos observed within 24 hours were discarded as non-specific deaths caused by injury or bacterial contamination. All embryos which died beyond 24 hrs post inoculation were regarded as suspicious. Inoculated eggs that had viable embryos between 24 and 120 hours were refrigerated overnight or freezed for a minimum of 4 hours to kill the embryo (Guy, 2005; Gelb and Jackwood, 2008; Sahar *et al*., 2015).

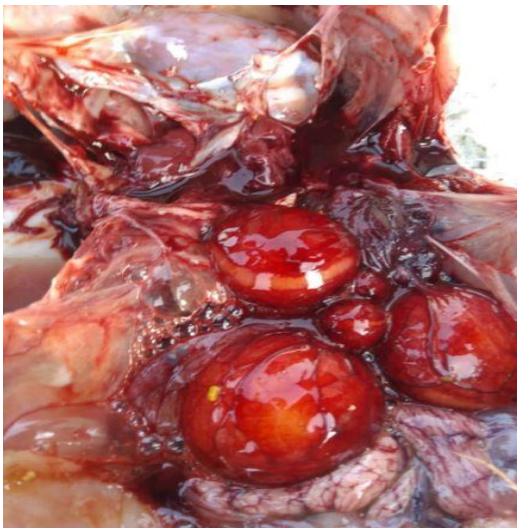
Microbiology

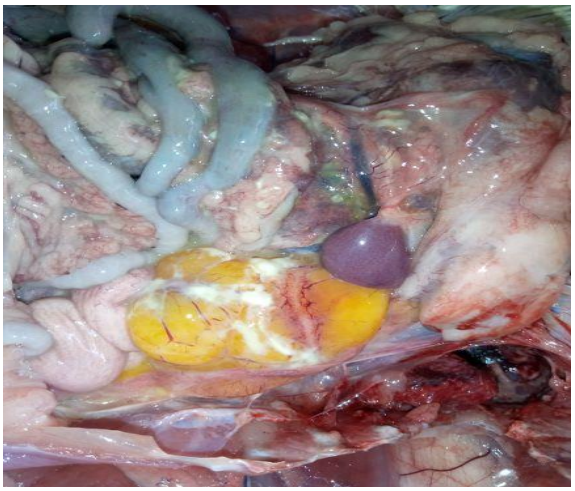
Swabs from the liver, lungs, kidneys, spleen and bone marrow were submitted to the microbiology laboratory for bacterial and fungal culture, the isolation and identification of the bacterial and fungal organism were carried out using standard microbiology methods (Zavala *et al*., 2008).

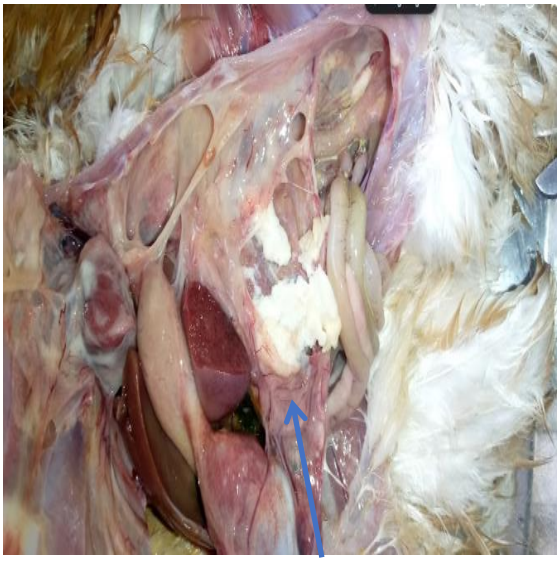
RESULTS Major clinical and gross pathological findings common to all the samples examined were pasty vent,

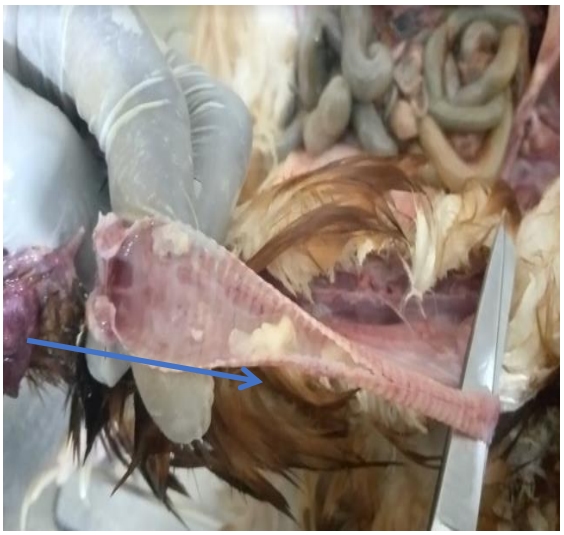
cyanotic combs and wattles, petechial haemorrhages on the abdominal and coronary fat, congestion of the ovarian follicles with caseous to egg yolk peritonitis, congested and oedematous lungs, congested to haemorrhagic trachea with mucopurulent to caseous exudate (Plate 1).











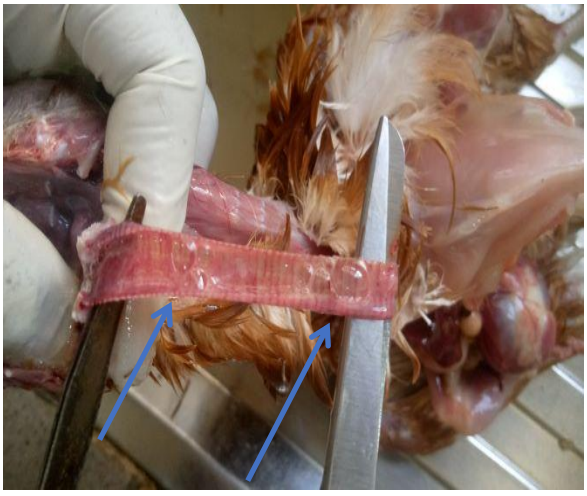


Plate I: (A) Cyanotic combs and wattles (B) Petechial hemorrhages on the abdominal fat (C) Congestion and hemorrhages in the ovarian follicles (D) Egg yolk peritonitis (E) Caseous peritonitis (F) Congested trachea with caseous exudate (G) Haemorrhagic trachea with mucoid exudate (H) Egg-shell defects in infected birds.

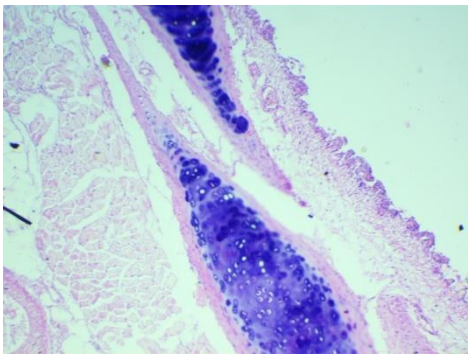


Plate II: Histopathology result

The histopathological lesion recorded was: Hyperaemia, oedema, necrosis and loss of cilia on the epithelial lining of the trachea with heterophils and lymphocytes infiltration of the lamina propria (Plate II.

Of the 20 sera tested, 17 (85%) were positive for avian influenza nucleoprotein H9 against indirect and competitive antibody capture ELISA (Table II). Antibodies were negative against the H5 and H7 subtypes.

Escherichia coli is the dominant bacteria isolated from the liver, spleen and bone marrow as well as *Klebsiella spp* from the liver and lungs from the farms monitored .

Low pathogenic avian influenza virus H9N2 were detected in six (6) samples by RT-qPCR and subsequently isolated in three of the six samples that tested positive for H9N2 by real-time RT-PCR using embryonated chicken egg (ECE).

TABLE II: Summary of Avian Influenza Indirect and Competitive Antibody Capture Elisa Results for H9 Virus Subtype from Affected Farms.

Sera Collection	H5			H7			H9		
	Total	Pos	%	Total	Pos	%	Total	Pos	%
Farm A	10	0	0	10	0	0	10	10	100
Farm B	10	0	0	10	0	0	10	7	70
G. total	20	0	0	10	0	0	20	17	85

KEY: Pos = positive

DISCUSSION

This study reports the detection and isolation of H9N2 LPAIV isolates from 2 commercial farms in Kano and Kaduna State. Following reported high mortality and, morbidity ranging from 18% to 40% (Table I). Highly pathogenic avian influenza subtype was ruled out from the result of the clinical pathological and microbiological examination.

The results of the molecular techniques (RT-qPCR) revealed that the farms were infected with the H9N2 LPAI virus. This corroborate with previous report of (Pillai *et al*., 2009; Swayne *et al*., 2008). where they opined that low pathogenic avian Influenza A virus such as H9N2 infection affect water and feed intake thereby causing a reduction in egg production in layer chicken. Similar finding were reported in 2019 from a live bird market in Nigeria forming two independent clusters with other LPAI H9N2 discovered in Africa as reported by (Suleiman *et al*., 2021).

The introduction of the LPAI virus (H9N2) subtype into North and West Africa may indicate that sub-regional commerce plays a key role in the virus's distribution, raising serious concerns about the virus's re-emergence.

There has been reports of H9N2 and H5NI virus recombination in China and Bangladesh (Dong *et al*., 2011; Chen *et al*., 2017; Monne *et al*., 2013). In Guangxi Zhuang China, H9N2 has been reported in dogs by (Li *et al.*, 2021) with 99.0 percent similarity to avian origin strains which raises serious public health concerns because of the greater affinity of this strain to mammalian (human) receptors and poses a serious threat to both humans and other mammal.

The isolation of *Escherichia coli* and *Klebsiella spp* in the farms is an indication that other bacteria co-infection may have contributed to the morbidity and mortality of infected birds. This is in agreement with the finding of (Peiris *et al*., 2007; Samaha *et al*., 2015) which indicated that

co-infection enhances the high level of harm to the chicken sector, disrupting the food chain and causing serious economic damage, according to this work. Antibodies against the H9 subtype of the AI virus were found in 17 (85%) of the 20 sera samples tested using antibody capture competitive and indirect ELISA (IDVet). This is a high prevalence compared to 10.4% reported by Aiki-Raji *et al.,* (2015) it is also higher than the prevalence of 22.0%, 2.0 and 78.0% reported by (Oluwayelu *et al.*, 2017) for LPAIV H5N2, H7N7 and H9N2 in Oyo state, Nigeria.

The prevalence in this study is higher than 12.9 percent reported by (Wakawa *et al*., 2012) in Kano State in 2012 because LPAI pattern of behaviour differs from that of HPAI and it is also possible to have 100% seroprevalence because of the low pathogenicity of the virus.

Also, in a related development (Abiayi *et al*., 2021) reported 26% prevalence to H9 subtype and 1.4% to H7, which are lower than the Prevalence recorded in this work.

The isolation of the H9N2 low pathogenic avian influenza (LPAI) virus from pooled tracheal and cloacal swabs collected from moribund birds inoculated in 9-11-day-old specific antibody negative (SAN) embryonated chicken eggs is important as previous attempts in England and Nigeria by (Parker *et al*., 2012) and (Aiki-Raj *et al*., 2015) were unsuccessful.

In view of the economic losses associated with avian influenza, continuous surveillance of avian influenza in Nigeria and other West African countries should be strengthened, and a One Health agenda should be adopted and implemented by countries within this region to mitigate economic losses, animal and human health.

CONCLUSION

These findings further confirmed the presence of the low pathogenic H9N2 subtype in Nigerian commercial layer farms, where co-infection with other diseases could cause catastrophic economic losses. Continuous avian influenza surveillance of all subtypes across the country to reduce the zoonotic and economic impact of avian influenza virus is advocated, particularly in this era of post COVID-19 pandemic and avian influenza reassortment with other species.

Acknowledgement

All the staff of Regional Laboratory for Animal Influenza and other Transboundary Animal Diseases; Kaduna Poultry Disease Laboratory (Olam Grains), Kaduna State, Nigeria; Animal Care Services Konsult Technical Laboratory, Abuja, Nigeria and Ambuvet Konsult, Kaduna State, Nigeria for their contribution towards the success of this work.

REFERENCES

Bello, K. O., Alebiosu, L. A., Lala, L.O., Irehore, O. T. and Oduguwa, O.O. (2015). Characteristics of Commercial Poultry and Spatial Distribution of Metabolic and Behavioral Diseases in Oyo State. *Sokoto Journal of Veterinary Sciences*,13(3):31

MacLachlan, N. J., Dubovi, E. J., Barthold, S. W., Swayne, D. E. and Winton, J. R. (2017). Fenner's Veterinary Virology. Academic Press, London, United Kingdom, 389 – 410

Capua, I. and Alexander, D.J. (2009). Avian Influenza and Newcastle Disease: A Field

and Laboratory Manual. *Springer*, Milan, Italia,1–186.

World Health Organization. Manual on Animal Influenza Diagnosis and Surveillance (**2002).** *World Health Organization*, Geneva, pp. 62.

Swayne, D.E. and Pantin-Jackwood, M. (2008). Pathobiology of avian influenza virus infections in birds and mammals. In: D.E. Swayne (Ed.), *Avian Influenza*. Blackwell Publishing, Ames, Iowa, 87–122.

Swayne, D.E., Suarez, D.L. and Sims, L.D. (2020). Influenza. In: Swayne, D. E ; Boulianne, M.;.Logue, K. M.;McDougald, R; Nair, V; Suarez,D. L (Eds), *Diseases of Poultry*, John Wiley and Sons, Inc, Hoboken, NJ, USA, 210–257.

Tombari, W., Nsiri, J., Larbi, I., Guerin, J. L., and Ghram, A. (2011). Genetic Evolution of Low Pathogenecity H9N2 Avian Influenza Viruses in Tunisia: Acquisition of New Mutations. *Journal of Virology*, 12(8), 467.

Abdel, H.S., Ellakany, H.F., Hussien, H.A., El-Bestawy, A.R.and Abdel, B.K.M. (2016). Pathogenicity of an Avian Influenza H9N2 Virus Isolated from Broiler Chickens in Egypt. *Alexandria Journal of Veterinary Science*, 51(2), 90 – 100.

El Houadfi, M., Fellahi, S., Nassik, S., Guérin, J. L. and Ducatez, M. F. (2016). First outbreaks and phylogenetic analyses of avian influenza H9N2 viruses isolated from poultry flocks in morocco. *Journal of Virology*, 13(1), 140. http://dx.doi.org/10.1186/s12985-016-05961

Zecchin, B., Minoungou, G., Fusaro, A., Moctar, S., Ouedraogo-Kabore, A., Schivo, A., Salviato, A., Marciano, S.and Monne, I. (2017). Influenza A (H9N2) virus, Burkina Faso. *Emerging Infectious Disease*, 23, 2118–2119.

Nagy, A., Mettenleiter, T. C. and Abdelwhab, E. M. (2017). A brief summary of the epidemiology and Genetic Relatedness of Avian Influenza H9N2 Virus in Birds and Mammals in the Middle East and North Africa. *Epidemiology and Infection*, 1 – 14.

Peacock, T. P., James, J., Sealy, J. E., and Munir, Iqbal. (2019). A Global Perspective on H9N2 Avian Influenza Virus. *Viruses*, 11 (7), 1-28.

Awuni, J.A., Bianco, A., Dogbey, O.J., Fusaro, A., Yingar, D.T., Salviato, A., Ababio, P.T., Milani, A., Bonfante, F. and Monne, I. (2019). Avian influenza H9N2 subtype in Ghana: Virus characterization and evidence of co-infection. *Avian Pathology*, 48(5), 470-476

Kariithi, H. M., Catharine, N. W, Helena, L. F, Elizabeth, A. P., Leonard, O. A., Yatinder, S. B., Auleria, A. A., Thomas, D. D., Claudio, L. A. and David, L. S. (2020). Genetic Characterization and Pathogenesis of the first H9N2 Low Pathogenic Avian Influenza Viruses Isolated from Chickens in Kenyan Live Bird Markets. *Infection, Genetics and Evolution*, 78, 1-9.

Maxime, F., Fidélia, D., Komla, B., Denis, K. B., Jeremy, C. J., Fred, W., Bernard, E., Gladys, A., Qouilazoni, A.U., Titus, T., Koffi, D., Komlan, A., Mvibudulu, N., Rachidatou, A.,

Trushar, J., Adam, R., Wolali, G., Ghazi, K., Pamela, M., Richard, J. W. and Mariette, F. D. (2021). Antigenic and Molecular Characterization of Low Pathogenic Avian Influenza A (H9N2) Viruses in Sub-Saharan Africa from 2017 through 2019. *Emerging Microbes and Infection*

http://dx.10(1):753-761
doi:10.1080/22221751.2021.1908097

Celia, A., Gerdes, G. H., Sinclair, M., Boto, W. G., James, P. K., Christina, E. B., Marco, R., Magdeline, D., Stefan, S., Graeme, S. C. and Adriaan, J. O. (2010). Phylogenetic Analysis of Influenza A Viruses (H6N8, H1N8, H4N2, H9N2, H10N7) Isolated from Wild Birds, Ducks, and Ostriches in South Africa from 2007 to 2009. *Avian Diseases*, 54 (1), 313 – 322

Mamadou, M. J., Amary, F., Mamadou, A. B., Boly, D., Sara, S., Déborah, G., Malick, F., Vincent E., Mbayame. and Ndongo, N.N. (2020). Genetic characterization of the first detected human case of low pathogenic avian influenza A/ H9N2 in sub-Saharan Africa, Senegal. *Emerging Microbes & Infections*, 9, 1092 – 1095.

Coker, T., Meseko, C. A., Odaibo, G. and Olaleye, D. (2014). Circulation of the Low Pathogenic Avian Influenza Subtype H5N2 Virus in Ducks at a Live Bird Market in Ibadan, Nigeria. *Infectious Diseases of Poverty* 3 **,** 38. https://doi.org/10.1186/2049-9957-3-38

Oluwayelu, D. O., Ayoyimika, O., Adebowale, I. A. and Aiki-Raji, C. O. (2017). Flock-Based Surveillance for Low Pathogenic Avian Influenza Virus in Commercial Breeders and Layers, Southwest Nigeria. *African Journal of Infectious Disease***.,** 11(1), 44-49.

Chinyere, C.N., Okwor, E. C., Meseko, C.A., Ezema, W.S., Choji, N.D., Amos, D.I., Sulaiman, L.K., Shittu, I. and Nwosuh, C. (2020). Sero-Detection of Avian Influenza A/H7 in Nigerian Live-Bird Markets in Plateau State. *Nig. Vet. J.* 41(2):161-174. https://dx.doi.org/10.4314/nvj.v41i2.7

Abiayi, D. C., Otolorin, G. R., Dzikwi-Emennaa, A. A. and Meseko, C. A. (2021). Serological Evidence of Influenza A/H9 in indigenous birds and level of awareness at live bird markets, Plateau State. *Sokoto Journal of Veterinary Sciences*, 19(4): 175-182. http://dx.doi.org/10.4314/sokjvs.v19i4.4

Suleiman, L., Shittu, I., Fusaro, A., Bitrus, I., Zecchin, B., Gado, D., Schivo, A., Bianco, A., Laleye, A., Gobbo, F., Vakuru, C., Joannis, T., Monne, I. and Meseko, C. A. (2021). Live Bird Markets in Nigeria: A Potential Reservoir for H9N2 Avian Influenza Viruses. *Viruses*, 13, 1445. https://doi.org/10.3390/v13081445

World Health Organisation. Annex 8.Viral transport media (VTM). (2006). In: Healing, T. (Ed.), Collecting, Preserving and Shipping Specimens for the Diagnosis of Avian Influenza A (H5N1) Virus Infection: Guide for Field Operations. *World Health Organization*, Geneva, pp. 42–43.

Spackman, E., Senne, D.A. and Suarez, D. L. (2002). Development of a Real-time Reverse Transcriptase PCR Assay for Type A Influenza Virus and the Avian H5 and H7

Hemaggluinin Subtypes. *Journal Clinical Microbiology* 40:3256-3260.

Isabella, M., Silvia, O., Annalisa, S., Cristian, D.B., Francesca, B., Angela, S., Alessandra, D., Bianca, Z., Ilaria, C. and Giovanni, C. (2008). Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9. *Avian Influenza Viruses*,1769–1773.

Sahar, A. E. R., Hoffmann, M., Lueschow, D., Eladl, A. and Hafez, H.M. (2015). Isolation and Characterization of New Variant Strains of Infectious Bronchitis Virus in Northern Egypt. *Adv Anim Veterinary Science*, 3, 362-371

Gelb, J. J. and Jackwood, M.W. (2008). Infectious Bronchitis, a Laboratory Manual for the Isolation, Identification and Characterization of avian pathogens (5th Edtn), American Association of Avian Pathologists, Florida, US.

Guy, J.S. (2005). Isolation and Propagation of Coronaviruses in embroynated Eggs, SARS- and Other Coronaviruses Laboratory Protocols.

Zavala, D. L., Swayne, D. E., Glisson, J. R., Pearson, J. E., Reed, W. M., Jackwood, M. W. and Woolcock, P. R.(2008). A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens. American Association of Avian Pathologists, Inc. Athens, Georgia.

Pillai, S.P., Pantin-Jackwood, M., Jadhao, S. J., Suarez, D.L. and Swayne, D.E. (2009). Pathobiology of Triple Teassortant H3N2 Influenza Viruses in Breeder Turkeys and its Potential Implication for Vaccine Studies in Turkeys. *Vaccine,* 27, 819–824.

Peiris, J. S. M., De Jong, M. D. and Guan, Y. (2007). Avian influenza (H5N1): a Threat to Human Health. *Clin. Microbiol. Rev*. 20, 243-267

Samaha, H., Ibrahim, M.S., Ayoub, M. and Shaaban, S.I.(2015). Seroepidemiology of Avian Influenza viruses H5 and H9 in Beheira Governorate. Alex. J. Vet. Sci. 44, 86-92.

Aiki-Raji, C. O., Adebowale, I. A., Victor, I A., Adetunji,S. A., Quadri, L., Adesina, M., Adekanye, G., Fatai, O., Olusegun, F. and Oluwayelu, D. O. (2015). *Asian Pac J Trop Dis*. 5 (5), 369-373.

Wakawa, A. M., Abdu, P. A., Oladele, S. B Sa'idu, L. and Mohammed, B. (2012). Risk Factors for the Occurrence and Spread of Highly Pathogenic Avian Influenza H5N1 in Commercial Poultry Farms in Kano, *Nigeria.Sokoto Journal of Veterinary Sciences*, 10(2), 40-51

Parker, C. D., Reid, S.M., Ball, A., Cox, W. J., Essen, S.C. and Hanna, A. l.(2012). First Reported Detection of a Low Pathogenicity Avian Influenza Virus Subtype H9 Infection in Domestic Fowl in England. *Vet Rec*, 171: 372

Kammon, A., Heidari, A., Dayhum, A., Eldaghayes, I., Sharif, M. and Monne, I. (2015). Characterization of Avian Influenza and Newcastle Disease Viruses from Poultry

in Libya. *Avian Dis*. 59, 422–30. http://dx.doi.org/10.1637/11068-032215-ResNote.

Dong, G.; Xu, C., Wang, C., Wu, B., Luo, J. and Zhang, H.(2011). Reassortant H9N2 Influenza Viruses Containing H5N1-like PB1 Genes Isolated from Black-billed Magpies in Southern China. *PLoS One*., 6:e25808. http://dx.doi.org/10.1371/journal.pone.0025808

Chen, L. J., Lin, X. D., Tian, J. H., Liao, Y., Ying, X. H. and Shao, J. W. (2017). Diversity, Evolution and Population Dynamics of Avian Influenza Viruses Circulating in the Live Poultry Markets in China. *Virology*, 505, 33–41. http://dx.doi.org/10.1016/j.virol.2017.02.009

Monne, I., Yamage, M., Dauphin, G., Claes, F., Ahmed, G. and Giasuddin, M. (2013). Reassortant Avian Influenza A(H5N1) Viruses with H9N2-PB1 Gene in Poultry, Bangladesh. *Emerg Infect Dis*, 19, 1630–1634. http://dx.doi.org/10.3201/eid1910.130534

41. Li, S., Sun, X. and Kong, Z. (2021). Whole Genome Sequencing Analysis of Canine H9N2 Subtype Influenza Virus Isolates from Guangxi and its Pathogenicity Experiments in Mice. *Chinese journal of Zoonoses*, 37(5), 398-403. Doi: 10.3969/j.issn.1002-2694.2021.00.061